

# Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron

## Is haemoglobin a biological Fenton reagent?

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The ability of oxyhaemoglobin and methaemoglobin to generate hydroxyl radicals ( $\text{OH}^\bullet$ ) from  $\text{H}_2\text{O}_2$  has been investigated using deoxyribose and phenylalanine as 'detector molecules' for  $\text{OH}^\bullet$ . An excess of  $\text{H}_2\text{O}_2$  degrades methaemoglobin, releasing iron ions that react with  $\text{H}_2\text{O}_2$  to form a species that appears to be  $\text{OH}^\bullet$ . Oxyhaemoglobin reacts with low concentrations of  $\text{H}_2\text{O}_2$  to form a 'reactive species' that degrades deoxyribose but does not hydroxylate phenylalanine. This 'reactive species' is less amenable to scavenging by certain scavengers (salicylate, phenylalanine, arginine) than is  $\text{OH}^\bullet$ , but it appears more reactive than  $\text{OH}^\bullet$  is to others (Hepes, urea). The ability of haemoglobin to generate not only this 'reactive species', but also  $\text{OH}^\bullet$  in the presence of  $\text{H}_2\text{O}_2$  may account for the damaging effects of free haemoglobin in the brain, the eye, and at sites of inflammation.

## INTRODUCTION

It now seems to be well established that formation of hydroxyl radicals ( $\text{OH}^\bullet$ ), or a similar highly oxidizing species, accounts for much of the damage done to biological systems by increased generation of superoxide radicals ( $\text{O}_2^{\bullet-}$ ) and  $\text{H}_2\text{O}_2$  (for reviews see [1–3]). Hydroxyl radical formation in systems generating  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  requires the presence of a suitable metal-ion promoter. Particular attention has been paid to low-molecular-mass iron complexes and to iron proteins as potential promoters of  $\text{OH}^\bullet$  formation *in vivo* [1,2]. The abilities of iron bound to ferritin [4,5], lactoferrin [6] and transferrin [6] to participate in  $\text{OH}^\bullet$  radical formation have been studied in detail. However, the ability of haemoglobin to accelerate  $\text{OH}^\bullet$  radical formation seems to be a subject of dispute.

Haemoglobin has long been known to stimulate lipid peroxidation [7–10], an effect which is partly due to the intact protein and partly due to release of iron ions from the protein by lipid peroxides [7,11]. However, the ability of haemoglobin to stimulate  $\text{OH}^\bullet$  radical formation is unclear. Early experiments failed to detect  $\text{OH}^\bullet$  when haemoglobin was added to  $\text{O}_2^{\bullet-}$ -generating systems [12], but an insensitive assay for  $\text{OH}^\bullet$  was used [13]. Eaton *et al.* [14] found that oxyhaemoglobin could oxidize dimethyl sulphoxide much more effectively than could methaemoglobin in the presence of  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ . The oxidation was inhibited by thiourea, and so was attributed to  $\text{OH}^\bullet$  formation [14]. However, thiourea is far from specific as a scavenger of  $\text{OH}^\bullet$  [15,16] and experiments with other scavengers were not reported. Benatti *et al.* [17] found that a methaemoglobin/ascorbate mixture could oxidize methional to ethene in a reaction inhibitable by mannitol, suggesting formation of  $\text{OH}^\bullet$ . In agreement with this, Gutteridge [11] found that incubation of commercial haemoglobin (presumably

largely methaemoglobin) with  $\text{H}_2\text{O}_2$  caused formation of  $\text{OH}^\bullet$ , as measured by the deoxyribose assay [18,19], and also a release of iron ions from the protein. However, he showed that the  $\text{OH}^\bullet$  formation was inhibited by desferrioxamine and transferrin, suggesting that it was mediated by the iron ions released from the protein by the  $\text{H}_2\text{O}_2$ , rather than by the haemoglobin molecule itself [11].

In the present paper, we report detailed studies on the ability of pure human oxyhaemoglobin and methaemoglobin to cause formation of  $\text{OH}^\bullet$  radicals from  $\text{H}_2\text{O}_2$  at pH 7.4. Two very different methods were used in an attempt to detect  $\text{OH}^\bullet$ , deoxyribose degradation [18,19] and aromatic hydroxylation of phenylalanine [20,21].

## MATERIALS AND METHODS

### Reagents

Desferrioxamine (Desferal) was a gift from CIBA-Geigy. All other reagents were of the highest quality available from Sigma or BDH Chemicals. Commercial human haemoglobin (found by us to be largely methaemoglobin, plus some degradation fragments) was oxidized with ferricyanide or reduced with a small excess of dithionite under aerobic conditions, and then purified by gel filtration on a 35 cm  $\times$  2.5 cm column of Sephadex G-15. Oxyhaemoglobin, methaemoglobin and hemichrome concentrations were calculated as in [22].

### Assays

Deoxyribose degradation [18,19] was measured essentially as described in [5]; full details are given in Table and Figure legends. Aromatic hydroxylation of phenylalanine was measured as described in [20] using the h.p.l.c. equipment and electrochemical detection described in [23].

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**Table 1. Deoxyribose degradation by methaemoglobin plus  $H_2O_2$** 

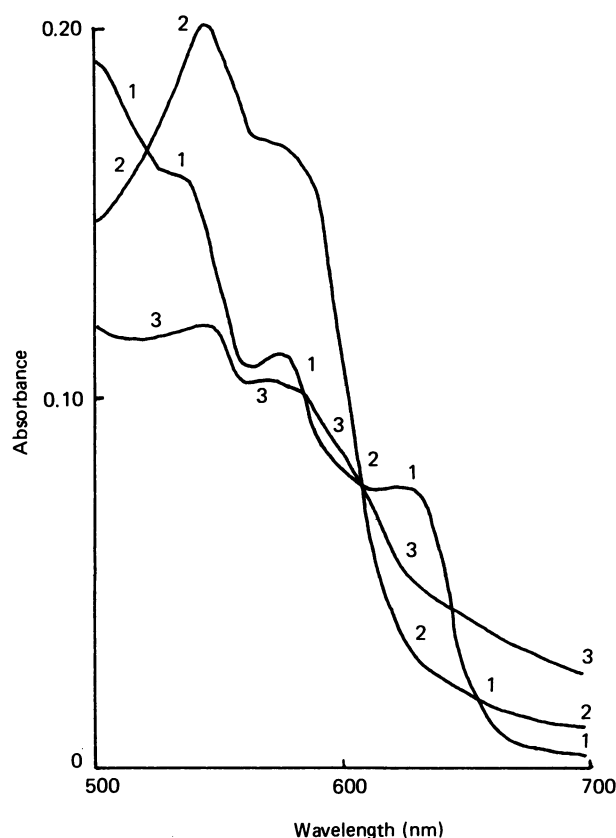
Reaction mixtures were incubated at 37 °C for 1 h. They contained the following reagents at the final concentrations stated: methaemoglobin (23  $\mu M$ -haem),  $H_2O_2$  (230  $\mu M$ ), deoxyribose (5.6 mM) and  $KH_2PO_4/KOH$  buffer, pH 7.4 (25 mM in phosphate). After incubation colour was developed, extracted into butan-1-ol and measured at 532 nm as described in [5]. Scavengers were added to the reaction mixture to give the final concentrations stated. Absorbances were read against a blank from which  $H_2O_2$  was omitted.

Reagent added to reaction mixture	$A_{532}$	Inhibition of deoxyribose degradation (%)
None (complete reaction mixture)	0.320	0
None ( $H_2O_2$ omitted)	0	100
100 $\mu M$ -Desferrioxamine	0.029	91
5 mM-Thiourea	0.042	87
5 mM-Urea	0.325	0
20 mM-Urea	0.329	0
20 mM-Mannitol	0.106	67
20 mM-Hepes	0.170	47
5 mM-Phenylalanine	0.154	52
5 mM-Salicylate	0.042	87
5 mM-Arginine	0.253	21
25 mM-Arginine	0.163	49
50 mM-Arginine	0.093	71
0.1 mM-Ascorbic acid	0.419	+31 (stimulation)
0.1 mM-Ascorbic acid + 0.1 mM-desferrioxamine	0.071	78

## RESULTS

### Hydroxyl radical generation by methaemoglobin

We began by investigating reports [11,17] that methaemoglobin generates  $OH^\bullet$  in the presence of  $H_2O_2$ . Deoxyribose, a hydrophilic molecule, was used as a detector. It is attacked by  $OH^\bullet$  to generate a product that, upon heating with thiobarbituric acid, forms a pink chromogen that can be measured at 532 nm. Table 1 shows that incubation of purified methaemoglobin with excess  $H_2O_2$  caused deoxyribose degradation. Deoxyribose reacts with  $OH^\bullet$  with a second-order rate constant of  $3.1 \times 10^9 M^{-1} \cdot s^{-1}$  [24]. The deoxyribose degradation could be inhibited by compounds that react with  $OH^\bullet$  at comparable or greater rates (mannitol [12], phenylalanine [20], salicylate [25,26], Hepes [27], arginine [28]), but not by urea, which reacts only slowly with  $OH^\bullet$  [12,29]. Deoxyribose degradation was also almost completely inhibited by low concentrations of desferrioxamine (100  $\mu M$  inhibited by 89–100% in a series of six experiments). Fig. 1 shows the absorption spectrum of the complete reaction mixture used in Table 1. Line 1 is the spectrum of methaemoglobin, which changes to line 2 when  $H_2O_2$  is added. Line 3 shows that, at the end of the experiment, the haem in the protein has been partially degraded and its absorbance lost. Inclusion of desferrioxamine in the reaction mixture, or of any of the  $OH^\bullet$  scavengers listed in Table 1, had no effect on the rate or extent of the haem degradation.

**Fig. 1. Absorption spectra recorded from the reaction mixtures listed in Table 2**

Line 1, no  $H_2O_2$  present (spectrum of methaemoglobin); line 2, spectrum immediately after adding  $H_2O_2$ ; line 3, spectrum after 60 min incubation at 37 °C showing significant haem loss.

Experiments similar to those in Fig. 1 and Table 1 were performed over a wide range of  $[haem]/[H_2O_2]$  ratios. Fig. 2 shows that the extent of deoxyribose degradation was proportional to the amount of haem destruction. Indeed, at 1:1  $[haem]/[H_2O_2]$  ratios, there was almost no haem destruction and very little deoxyribose degradation. In all these experiments, the pattern of inhibition by  $OH^\bullet$  scavengers and by desferrioxamine was very similar to that shown in Table 1.

Deoxyribose degradation by methaemoglobin/ $H_2O_2$  mixtures was, as expected [5], stimulated by addition of ascorbic acid to the reaction mixture, and the increased rate of deoxyribose degradation was inhibited by desferrioxamine (Table 1).

To confirm the generation of  $OH^\bullet$  radicals by methaemoglobin/ $H_2O_2$  mixtures, a completely different (aromatic and hydrophobic) detector molecule for  $OH^\bullet$  was used. Phenylalanine reacts with  $OH^\bullet$  ( $k_2$  approx.  $1.9 \times 10^9 M^{-1} \cdot s^{-1}$  [29]), eventually generating three isomeric tyrosines: *o*-tyrosine (2-hydroxyphenylalanine), *p*-tyrosine (4-hydroxyphenylalanine) and *m*-tyrosine (3-hydroxyphenylalanine) (Fig. 3 [20,21,30]). Hydroxylation of phenylalanine required the simultaneous presence of methaemoglobin and excess  $H_2O_2$  (sufficient to ensure some haem degradation). Hydroxylation was inhibited by 'scavengers' that react with  $OH^\bullet$  with rate constants equal to or greater than that of phenylalanine (Table 2).

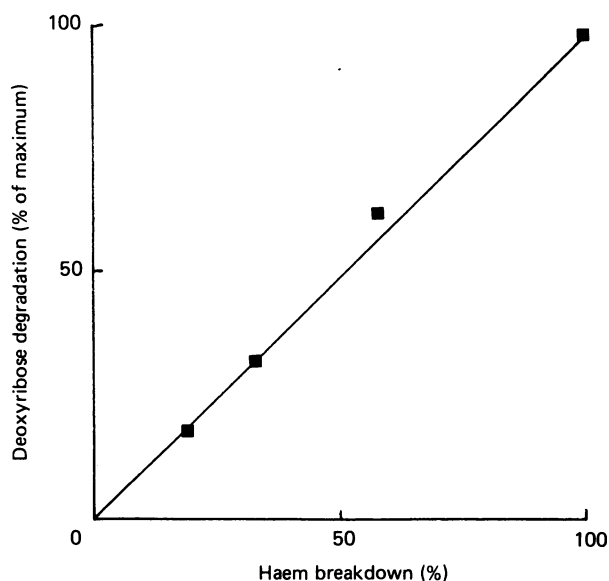


Fig. 2. Relationship of haem destruction to deoxyribose degradation in methaemoglobin/ $\text{H}_2\text{O}_2$  mixtures

Deoxyribose degradation at different [methaemoglobin]/ $[\text{H}_2\text{O}_2]$  ratios was measured as described in Table 1. Extent of haem degradation was calculated using the absorption spectra in Fig. 1. The extent of haem loss shown in line 3 of Fig. 1 is taken as 100%.

In the presence of the scavenger salicylate, phenylalanine hydroxylation was inhibited (Table 2), and new peaks were seen on the h.p.l.c. chromatogram at the retention times expected for 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate, which are products of attack of  $\text{OH}^\cdot$  on the salicylate molecule [26]. Phenylalanine hydroxylation was also inhibited by desferrioxamine (Table 2).

### Experiments with oxyhaemoglobin

Oxyhaemoglobin was generated by careful reduction of methaemoglobin with a slight excess of dithionite, followed by purification using gel filtration under aerobic conditions. Fig. 4 shows the time course of deoxyribose degradation promoted by oxyhaemoglobin plus  $\text{H}_2\text{O}_2$ . When  $\text{H}_2\text{O}_2$  is in excess over haem on a molar basis, there is an initial rapid 'burst' of deoxyribose degradation, followed by a lower rate (Fig. 4, upper line). However, when  $\text{H}_2\text{O}_2$  is equimolar to the haem present, only the rapid 'burst' is seen (Fig. 4, lower line). Fig. 5 shows the absorption spectra of the reaction mixtures at zero time and after 2 min and 60 min. At a 1:1 [oxyhaem]/ $[\text{H}_2\text{O}_2]$  ratio ( $21 \mu\text{M}$   $\text{H}_2\text{O}_2$ ), addition of  $\text{H}_2\text{O}_2$  caused minor changes in the absorption spectrum after 2 min (line B). After 60 min (line C) there was a mixture of oxyhaemoglobin, methaemoglobin and some hemichrome, but no apparent haem destruction. However, a large excess of  $\text{H}_2\text{O}_2$  (10:1 ratio,  $210 \mu\text{M}$ - $\text{H}_2\text{O}_2$ ) caused rapid methaemoglobin formation and haem destruction (line E).

Experiments carried out with  $\text{OH}^\cdot$  scavengers and desferrioxamine in reaction mixtures incubated for 1 h with a 10-fold excess of  $\text{H}_2\text{O}_2$  over oxyhaemoglobin haem gave results comparable with those in Table 1, which is perhaps not surprising in view of the rapid methaemoglobin formation and haem destruction

(Fig. 5). However, when experiments were carried out to study the initial 'burst' of deoxyribose degradation (2 min incubation, 1:1  $[\text{H}_2\text{O}_2]/[\text{oxyhaem}]$  ratio), different results were obtained (Table 3). The 'burst' of deoxyribose degradation was not inhibited by salicylate and phenylalanine, at concentrations that would compete with deoxyribose for  $\text{OH}^\cdot$ , and it was only weakly inhibited by arginine. Inhibition by mannitol was less than expected. However, the deoxyribose degradation was inhibited by urea, which scavenges  $\text{OH}^\cdot$  radicals poorly [12,29], and the inhibition by Hepes was greater than expected. A comparison of the results in the two 'inhibition' columns of Table 3 strongly suggests that, if the deoxyribose-degrading species produced by methaemoglobin plus excess  $\text{H}_2\text{O}_2$  is taken to be  $\text{OH}^\cdot$  (previous section), then the deoxyribose-degrading species produced by oxyhaemoglobin plus limited  $\text{H}_2\text{O}_2$  is not  $\text{OH}^\cdot$ . Further evidence for this conclusion is that no hydroxylated products were detected when phenylalanine was incubated with the oxyhaemoglobin/ $\text{H}_2\text{O}_2$  mixture described in Table 3, even though the electrochemical detector used is sufficiently sensitive to detect the three tyrosines corresponding to that amount of  $\text{OH}^\cdot$  that would cause deoxyribose degradation equivalent to that produced by oxyhaemoglobin plus limited  $\text{H}_2\text{O}_2$  (the 'burst' in Fig. 4).

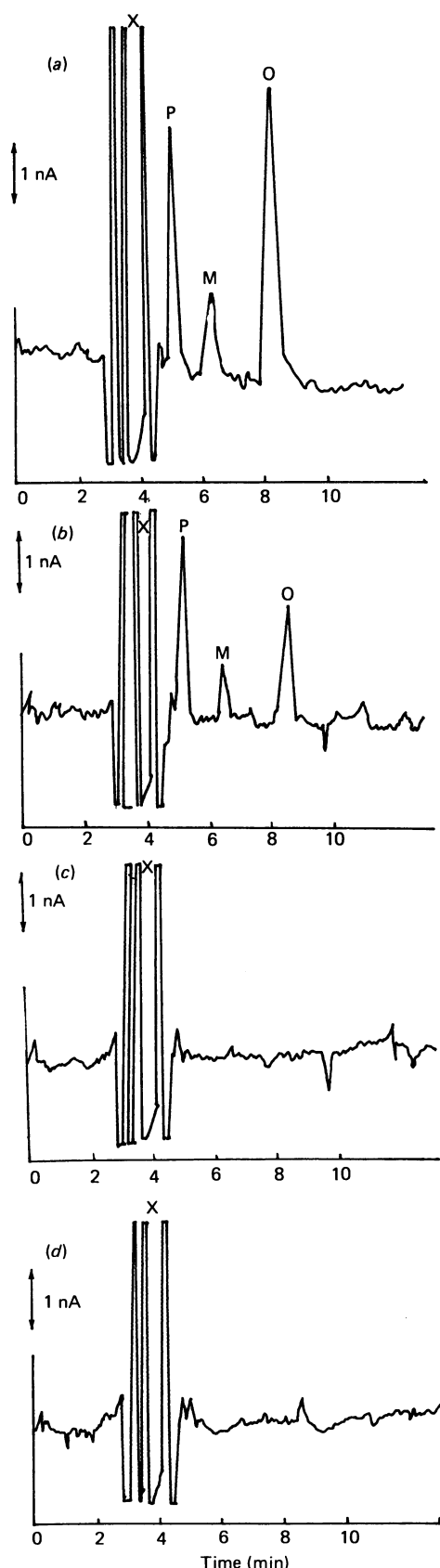
### DISCUSSION

Oxyhaemoglobin is rapidly oxidized by peroxides, and methaemoglobin is degraded with the apparent release of iron ions. Hence studies designed to answer the question 'is haemoglobin a Fenton reagent?' must consider what is happening to the protein in the reaction mixture. Our studies with methaemoglobin plus  $\text{H}_2\text{O}_2$  support the proposals of Gutteridge [11] that  $\text{H}_2\text{O}_2$  (in excess) decomposes haem and releases iron ions from the protein. These then react with  $\text{H}_2\text{O}_2$  to form  $\text{OH}^\cdot$  in Fenton-type reactions. Identification of the deoxyribose-degrading species as  $\text{OH}^\cdot$  is strengthened by its ability to hydroxylate

Table 2. Hydroxylation of phenylalanine by methaemoglobin plus  $\text{H}_2\text{O}_2$

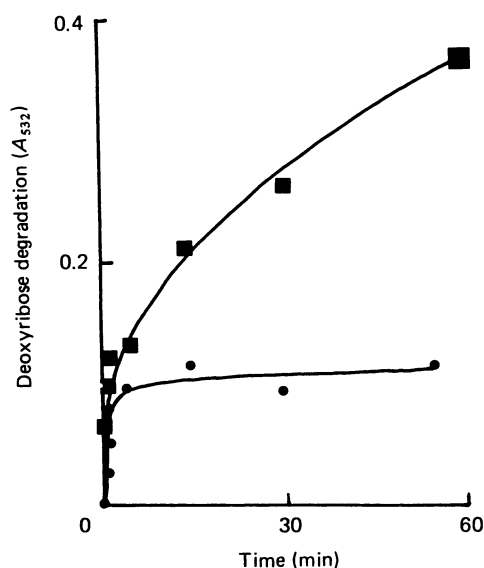
Reaction mixtures were as described in the legend to Fig. 3. 'Total hydroxylated products' means the sum of the amounts of *o*-, *p*- and *m*-tyrosines produced. Reagents were added to the reaction mixtures to give the final concentrations stated.

Reagent added to reaction mixture	Total hydroxylated products formed (nmol)	Inhibition of phenylalanine hydroxylation (%)
None (complete reaction mixture)	1.62	—
None (methaemoglobin omitted)	0.13	92
None ( $\text{H}_2\text{O}_2$ omitted)	0.00	100
100 $\mu\text{M}$ -Desferrioxamine	0.21	87
5 mM-Urea	1.54	5
20 mM-Mannitol	0.39	76
5 mM-Salicylate	0.06	96
50 mM-Arginine	0.40	75



**Fig. 3. Hydroxylation of phenylalanine by a methaemoglobin/ $\text{H}_2\text{O}_2$  mixture**

Reaction mixtures (final vol. 1 ml) contained the following reagents at the final concentrations stated: methaemoglobin ( $23 \mu\text{M}$ -haem);  $\text{H}_2\text{O}_2$  ( $230 \mu\text{M}$ ); phenylalanine



**Fig. 4. Deoxyribose degradation by oxyhaemoglobin plus  $\text{H}_2\text{O}_2$**

Deoxyribose degradation was measured as described in the legend to Table 1, except that methaemoglobin was replaced by oxyhaemoglobin (final concentration  $21 \mu\text{M}$ , as haem) and the final  $[\text{H}_2\text{O}_2]$  was  $21 \mu\text{M}$  (●) or  $210 \mu\text{M}$  (■). Absorbances were read against a blank from which  $\text{H}_2\text{O}_2$  was omitted.

phenylalanine (giving three isomeric tyrosines) and salicylate (giving two isomeric dihydroxybenzoates) and its scavenging by all the  $\text{OH}^\bullet$  scavengers tested. Hence the studies in [17] were most probably looking at  $\text{OH}^\bullet$  produced by iron ions released from methaemoglobin by  $\text{H}_2\text{O}_2$ . Ascorbate would accelerate  $\text{OH}^\bullet$  formation by redox-cycling the iron [5]. If insufficient  $\text{H}_2\text{O}_2$  to degrade methaemoglobin is used, little (if any) deoxyribose degradation or phenylalanine hydroxylation is observed (Fig. 2).

Our results with oxyhaemoglobin are more complex. Firstly, if experiments use a large excess of  $\text{H}_2\text{O}_2$  over oxyhaemoglobin and a long incubation time, any contribution by oxyhaemoglobin itself to generation of a 'reactive species' will be overshadowed by the formation of methaemoglobin and its release of iron ions (Fig. 4). However, our experiments suggest that mixing of oxyhaemoglobin and  $\text{H}_2\text{O}_2$  rapidly generates a reactive species that can degrade deoxyribose to thiobarbituric

(5 mM);  $\text{KH}_2\text{PO}_4/\text{KOH}$  buffer, pH 7.4 (25 mM in phosphate). After incubation at  $37^\circ\text{C}$  for 1 h, reaction mixtures were centrifuged through Amicon Centrifree micro partition devices, to remove protein. Samples were injected into the h.p.l.c. equipment described in [23], with electrochemical detection at 0.82 V. The eluent was 88% (v/v) 30 mM-sodium citrate/27.7 mM-sodium acetate buffer, pH 4.75, and 12% (v/v) methanol at a flow rate of 1 ml/min, continuously sparged with helium. X, buffer peaks. (a) Separation of standard mixture of *o*-, *m*- and *p*-tyrosines (O, P and M respectively) ( $1 \mu\text{M}$  each). (b) Analysis of reaction mixture containing methaemoglobin plus  $\text{H}_2\text{O}_2$ . (c) As (b), but with no  $\text{H}_2\text{O}_2$  added. (d) As (b), with no methaemoglobin added.

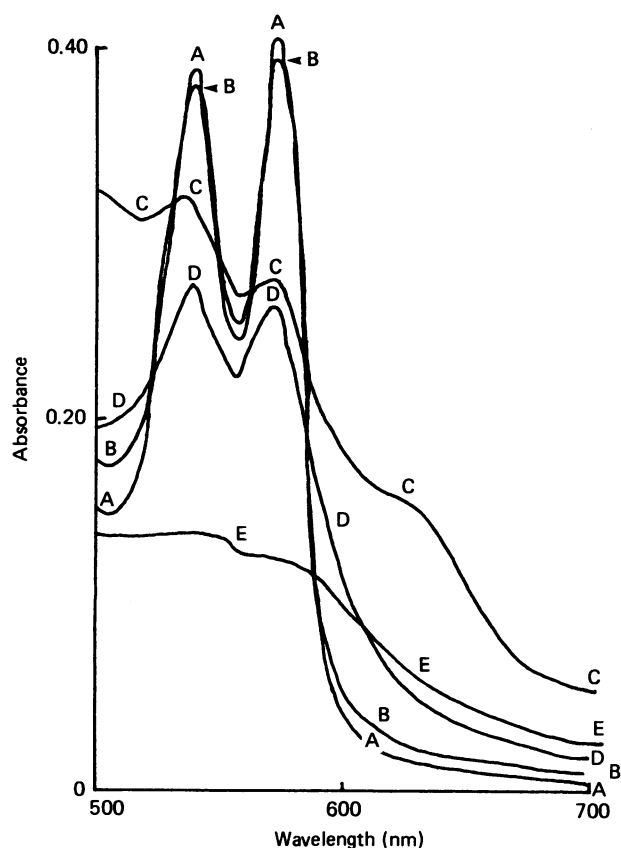


Fig. 5. Absorption spectra of the reaction mixtures in Fig. 4

Line A,  $\text{H}_2\text{O}_2$  omitted (spectrum of oxyhaemoglobin); line B,  $21 \mu\text{M-H}_2\text{O}_2$  2 min after  $\text{H}_2\text{O}_2$  addition; line C,  $21 \mu\text{M-H}_2\text{O}_2$  60 min after  $\text{H}_2\text{O}_2$  addition; line D,  $210 \mu\text{M-H}_2\text{O}_2$  2 min after  $\text{H}_2\text{O}_2$  addition; line E,  $210 \mu\text{M-H}_2\text{O}_2$  60 min after  $\text{H}_2\text{O}_2$  addition.

acid-reactive material. This species does not seem to be  $\text{OH}^\cdot$ , since it does not hydroxylate phenylalanine, and it is not well-scavenged by salicylate, arginine or phenylalanine (Table 3). On the other hand, it seems more reactive to urea and Hepes than is  $\text{OH}^\cdot$  (Table 3). The species may involve iron, since there is partial inhibition by desferrioxamine, although a direct scavenging by this molecule cannot be ruled out [2]. It is possible that a ferryl species is formed, since ferryl seems less reactive than  $\text{OH}^\cdot$  to scavengers such as arginine [28]. Indeed, the existence of 'reactive species' additional to  $\text{OH}^\cdot$  in biological systems has been a hotly debated topic [1,2,28,31,32]. The reactive species formed in our experiments is accessible to deoxyribose, mannitol, Hepes and urea, and its formation seems to cease after 2 min, even though further changes in the spectrum of the protein occur after that time (Fig. 5). The first three of these scavengers are hydrophilic molecules, especially as deoxyribose and mannitol have extensive hydrogen bonds to water. They are thus very unlikely to enter the hydrophobic haem pocket of haemoglobin, which suggests that the 'reactive species' is actually released from the protein. Its nature requires further investigation. Our results also mean that deoxyribose degradation is not in itself a specific detector for  $\text{OH}^\cdot$ . The role of  $\text{OH}^\cdot$  must be established by the use of appropriate scavengers, as has been stressed previously [5,18–20].

Haemoglobin is normally safely 'compartmentalized' in erythrocytes, which are rich in antioxidant defence enzymes. Free haemoglobin is toxic [33], e.g. to the brain [34], to the eye [35] and at sites of inflammation [36]. The ability of oxyhaemoglobin to generate a reactive oxidant species in the presence of low concentrations of  $\text{H}_2\text{O}_2$ , and the ease with which methaemoglobin releases iron in a form that can stimulate lipid peroxidation and  $\text{OH}^\cdot$  radical formation, provide a logical explanation of this toxicity [37,38].

Table 3. Deoxyribose degradation by oxyhaemoglobin plus  $\text{H}_2\text{O}_2$

Experiments were performed as described in the legend to Table 2 except that methaemoglobin was replaced by oxyhaemoglobin ( $21.7 \mu\text{M-haem}$ ),  $[\text{H}_2\text{O}_2]$  was  $21.7 \mu\text{M}$ , and incubation at  $37^\circ\text{C}$  was for 2 min only. Reagents were added to give the final concentrations stated. The final column shows, for comparison, the inhibitions produced in the methaemoglobin/ $\text{H}_2\text{O}_2$  system described in Table 2.

Reagent added to reaction mixture	Deoxyribose degradation		
	$A_{532}$	Inhibition (%) (Column A)	Inhibition in methaemoglobin/ $\text{H}_2\text{O}_2$ system (Table 1) (%) (Column B)
None (complete reaction mixture)	0.102	0	—
None ( $\text{H}_2\text{O}_2$ omitted)	0.000	100	—
100 $\mu\text{M-Desferrioxamine}$	0.045	56	91
5 mM-Thiourea	0.010	90	87
5 mM-Urea	0.066	35	0
10 mM-Urea	0.063	38	0
20 mM-Urea	0.047	54	0
20 mM-Mannitol	0.050	51	67
20 mM-Hepes	0.000	100	47
5 mM-Phenylalanine	0.107	0	52
5 mM-Salicylate	0.109	0	87
5 mM-Arginine	0.098	4	21
25 mM-Arginine	0.091	11	49
50 mM-Arginine	0.083	19	71

B.H. thanks the Arthritis and Rheumatism Council, and the Wellcome Trust for financial support. A.P. is a NATO grant recipient and B.H. is a Lister Institute Research Fellow. We are very grateful to Dr. H. Kaur for help with the h.p.l.c. studies.

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Received 10 June 1987/13 August 1987; accepted 18 September 1987